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Involvement of soluble proteinous factors in auxin-induced modulation of P-type ATPase in rice (*Oryza sativa* L.) seedlings

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Abstract The marked difference in auxin sensitivity between plant roots and shoots was studied in terms of auxin-induced stimulation of membrane P-type ATPase. The results suggest the existence in rice seedlings of, at least, two isoforms of soluble proteinous factors (SPF), $\mathbf{SPF_I}$ and $\mathbf{SPF_{II}}$, which are involved in the stimulatory action of auxin on the enzyme. It also is indicated that $\mathbf{SPF_I}$ which mediates the auxin effect in low hormone concentration range ($\mathbf{10^{-10}}$–$\mathbf{10^{-7}}$ M IAA) is the dominant isoform in roots, whereas $\mathbf{SPF_{II}}$ which does in high hormone concentration range ($\mathbf{10^{-7}}$–$\mathbf{10^{-4}}$ M IAA) is that in shoots.

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Key words: Auxin sensitivity; Dose response curve; Indole-3-acetic acid; P-type ATPase; Soluble proteinous factor

1. Introduction

Auxin modulates ATP hydrolysis activity of the plasma membrane as well as its proton-translocation activity in various plant materials [1–7]. However, the mode of action by which auxin regulates the membrane H^+-ATPase activity remains to be determined. There is evidence for that auxin-induced stimulation of proton-pumping activity of the membrane correlates with increased affinity of the H^+-ATPase for its substrate [2,3,6]. Since this affinity change does not involve direct auxin effects on the enzyme itself [8,9], certain indigenous factor(s) may be assumed to be involved in the auxin signal perception at the membrane. Related to this, a number of experimental approaches concern the membrane-associated auxin binding protein (ABP1), which might either be the putative auxin receptor [10,11] or associate with specific plasma membrane proteins to allow formation of active auxin signal-transducing units [5].

ABP1 has been indicated to enhance the sensitivity of plant protoplasts to auxin, as accessed by transmembrane potential difference [5]. Taken tobacco mesophyll protoplasts, for instance, they normally have the optimum auxin sensitivity of 10^{-5}–10^{-6} M, which is gradually increased by increasing addition of ABP1 from maize, up to 10^{-9}–10^{-10} M by 1 nM of maize ABP. Therefore, the well known high sensitivity of root growth responding to exogenous auxin could be ascribed to high content of ABP1 in roots. The experiments of Radermacher and Klämbt with corn seedlings [12], however, failed to show that roots contain much more ABP compared with the coleoptiles. Instead, these workers were able to demonstrate the existence of ABP isoforms in corn root tips, whose dissociation constants for naphthalene acetic acid are somewhat – by ca. 1 to 2 orders of magnitudes – lower than those from the coleoptiles.

In the present study, we found that P-type ATPase activity of microsomal membranes from rice (*Oryza sativa* L.) seedlings responds biphasically to auxin only in the presence of certain soluble proteinous factors (SPF), which are, unlike the membrane-associated ABP1, readily removed from the membrane preparations by washing. This paper demonstrates that there exist, at least, two isoforms of SPF in rice, tentatively designated as SPF_I, that mediates the ATPase stimulation by auxin in low concentration range (10^{-10}–10^{-7} M IAA), and SPF_{II}, that does in high concentration range (10^{-7}–10^{-4} M IAA). Our results also indicate that the dominant isoform of SPF in roots is SPF_I, whereas that in shoots is SPF_{II}. These observations may provide an explanation for the marked difference in in vivo auxin sensitivity between plant shoots and roots.

2. Materials and methods

Rice seedlings were grown for one week in an incubator without illumination at 26°C. Shoots and roots were harvested separately and stored at −80°C until used.

About 50 g of plant samples were powdered in liquid nitrogen, homogenized with 2 to 3 volumes of an extraction buffer (0.25 M sucrose, 3 mM EDTA, 2.5 mM dithiothreitol and 25 mM Tris-Mes, adjusted to pH 6.7) and filtered through 4 layers of cotton gauze. The filtrate was centrifuged at 12 000×g for 15 min to obtain supernatants, referred to as crude extracts. Microsomal membrane precipitates were prepared by ultra-centrifuging crude extracts at 85 000×g for 1 h and washed by resuspending in a small volume of a washing buffer (0.25 M sucrose, 3 mM $MgSO_4$ and 30 mM Tris-Mes, adjusted to pH 6.5) and subsequently recentrifuging at 85 000×g for 30 min. 'Soluble protein' was prepared from the supernatants of the first ultracentrifugation of crude extracts by ammonium sulfate (30 to 60%) fractionation, followed by a gel permeation chromatography using a Sephadex G-25 column (1×20); the protein fractions eluted in the void volume were collected.

ATPase activity was assayed by a coupled enzyme method, in which the pyruvate kinase and lactate dehydrogenase reactions were coupled to the production of ADP [13], as follows. Preincubating 1.9 ml of a mixture containing the sample to be assayed, 2 units of pyruvate kinase and 2 units of lactate dehydrogenase in an assay buffer (0.01% Triton X-100, 50 mM KCl, 1 mM NaN_3, 1 mM Na-molybdate, 200 mM KNO_3, 0.2 mM NADH, 0.2 mM phosphoenolpyruvate and 20 mM Tris-Mes, adjusted to pH 6.5) at 37°C for 5 min in either the presence of Na-orthovanadate (0.5 mM) or its absence, 100 μl of 6 mM K-ATP, kept at 37°C, were added to start the reaction. NADH oxidation catalyzed by lactate dehydrogenase was followed spectrophotometrically at 340 nm. The activity of P-type ATPase was calculated from the slope of the NADH oxidation kinetics measured in the presence of orthovanadate and that measured in its absence, subtracting the former from the latter.

Proteins were quantified by the Bradford method with bovine serum albumin as the standard [14].

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3. Results

Membrane-associated P-type ATPase was assayed in crude extracts from shoots and roots of rice seedlings and changes in the enzyme activity responding to applied indole-3-acetic acid at varied concentrations (0–10^{-4} M) were measured. As shown in Fig. 1, the vanadate-sensitive ATPase in the shoot extracts was maximally stimulated by IAA of 5×10^{-6} M while the largest stimulatory effect of auxin on the enzyme was seen at 5×10^{-9} M IAA in the root extracts. The dose response curves for the auxin action could be said to be bell-shaped at large.

Fig. 2 shows that microsomal membranes, when washed twice with the washing buffer, carried only small residual activity of P-type ATPase, which was not affected by the addition of IAA; in contrast, unwashed membrane preparations bore somewhat increased activity, which was further enhanced by the IAA application.

Partially purified soluble protein of crude extracts from either shoots or roots, ammonium sulfate-fractionated and subsequently gel-filtrated, was mixed with the washed microsomal membranes and then checked whether its presence is required for the auxin action on microsomal P-type ATPase. As expected, the results shown in Fig. 3 indicate that certain unknown components of the soluble protein mediate the auxin effect in the membranes; furthermore, the dose response curves showed a close similarity to those measured with crude extracts.

Even in the absence of exogenous auxin, the addition of the soluble protein per se brought about an increase in the vanadate-sensitive ATPase activity of microsomes, which was assumed to result from binding of some soluble protein components to the ATPase. In order to ascertain this, we measured the dependence of the ATPase activation on the concentration of added protein and obtained the corresponding Hill plot. From the plot shown in Fig. 4, the Hill coefficient measured in a system consisting of microsomes and the soluble protein, both of which were from the shoot extracts, was 0.97 ± 0.11. Practically identical observation was made in the root-originated microsomes and soluble protein mixtures (data not shown). The soluble protein itself did not show any detectable ATPase activity, apparently not contributing to the increased activity in the microsomes-soluble protein systems.

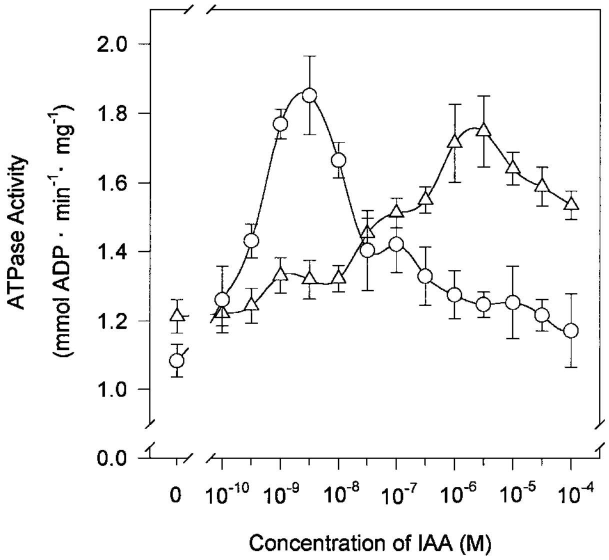

Fig. 1. Effect of IAA on P-type ATPase in crude extracts from roots (○) and shoots (△) of rice seedlings. The activity is expressed by the rate of ADP production per mg membrane-protein. Results are means of five separate experiments, showing the standard errors with error bars.

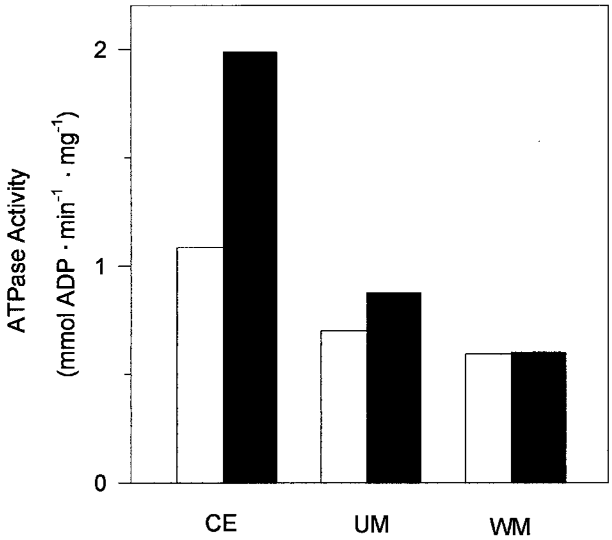

Fig. 2. Changes in P-type ATPase activity, in the presence of 1×10^{-5} M IAA (black bars) and its absence (white bars), with the progress of isolation of microsomal membranes from rice shoots. Results are means of three separate experiments. CE, crude extracts; UM, unwashed membranes, WM, washed membranes.

4. Discussion

Auxin regulates some physiological activities of plants in biphasic manners depending on its applied concentration, as has been demonstrated by elongation growth of the coleoptiles and root segments of corn [12,15] as well as by proton translocation activity of tobacco plasma membranes [4,5]. Increasing the auxin concentration at first gives rise to stimulatory effects but a point is then reached where a further increase in auxin supply leads to the disappearance of the stimulation, sometimes even to the occurrence of inhibitory effects. Such typical response to the phytohormone is also seen in P-type ATPase of microsomal membranes from rice seedlings.

It is well known that plant roots are much more – up to 10 000 times – sensitive to auxin than shoots [16]. We consider that our results disclose an aspects of biochemical basis of this marked difference in auxin sensitivity between them (Figs. 1 and 3). Further, the observation that the presence of certain soluble protein components is a prerequisite for substantiating the auxin effect in microsomal membranes (Figs. 2 and 3) is pertinent to the concept of soluble receptor proteins mediating various actions of auxin [17–19]. In this respect, those protein components could be soluble ABP. Because we have not yet examine whether they bind auxin with reasonably high affinity, however, for the moment we would refer to them as soluble proteinous factors (SPF).

It appears that there exist at least two isoforms of SPF in rice seedlings, $\mathrm{SPF_I}$ which is involved in the stimulatory effect of auxin in low concentration range (10^{-10}–10^{-7} M IAA) and $\mathrm{SPF_{II}}$ which is responsible for that in high concentration range (10^{-7}–10^{-4} M IAA). In addition, our results indicate that the dominant isoform of SPF in roots is $\mathrm{SPF_{II}}$ while that in shoots is $\mathrm{SPF_{II}}$ (Fig. 3). Notwithstanding, both isoforms seem to be present in roots as well as in shoots. With regard to this, note that there are small peaks or, at least, shoulder regions besides the major bell-shaped peaks on the respective dose response curves for the auxin effect in the membranes-soluble protein systems (Fig. 3). The shoulder displayed by a system containing the soluble protein from shoots arises in the concentration range of IAA where the maximum stimulation occurs in the case that the root soluble protein is present and vice versa. It would further be suggestive that the marked difference in auxin sensitivity of the membrane ATPase activity between roots and shoots is likely due to the difference in the properties of the respective dominant SPF isoforms involved, regardless of tissue sources of the membranes (Fig. 3).

Since auxin exerts its effects on P-type ATPase in rice microsomes only via SPF, the possibility would not be ruled out that these proteinous factors have the ability, independently of IAA, to bind to the membrane-associated enzyme, modulating to a certain extent the enzyme activity through protein-protein interaction. This conjecture seems to be in line with the observation that the soluble protein contains some components which activate the ATPase without cooperativity by binding at 1:1 ratio, as clearly indicated by the Hill coefficient (Fig. 4). In fact, a similar observation has been made with microsomal membranes and soluble protein preparations from oat shoot pulvini, in that soluble ABP stimulates the vanadate-sensitive ATPase activity of the membranes even in the absence of indole-3-acetic acid [7]. Despite such putative function of SPF as an activator of plant membrane P-type ATPase, its major physiological relevance may rather be found in the role as the mediator for auxin-induced modulation of the activity of H^+-translocation across membranes in plants

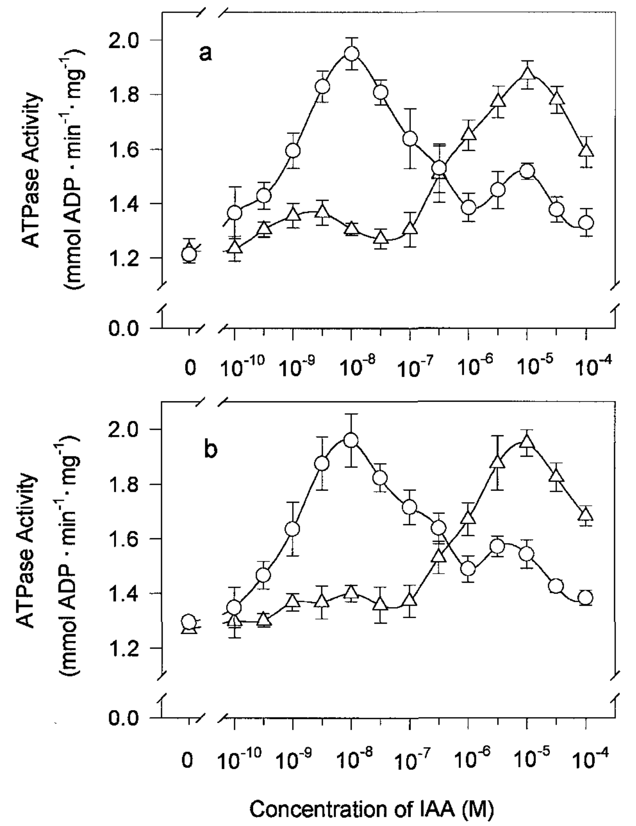

Fig. 3. Dose response curves for IAA-induced stimulation of P-type ATPase activity of microsomes from shoots (a) and roots (b) in the presence of 'soluble protein' from roots (○) and shoots (△). Reaction mixtures used for the ATPase assay contained 50 µg/ml of the soluble protein, 20 µg protein/ml of microsomes and IAA ($0 \sim 1 \times 10^{-4}$ M) in the assay buffer. Data are presented as mean ± SE ($n = 3$).

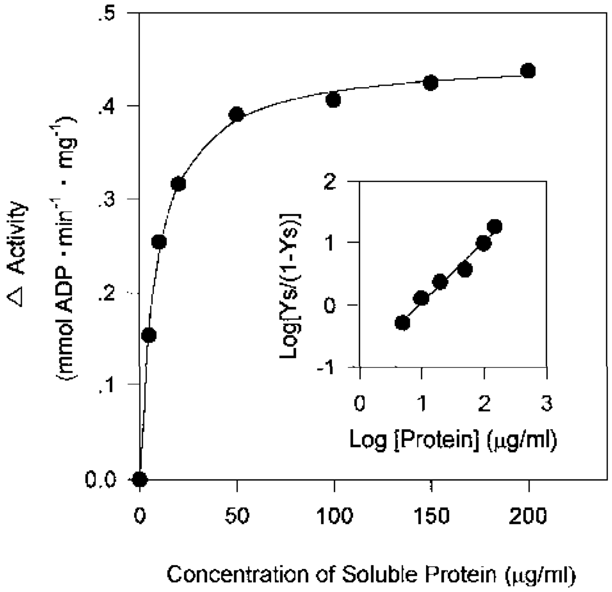

Fig. 4. Increase in ATPase activity (△, activity) of microsomes prepared from rice shoots with increased concentration of the shoot soluble protein. ATPase activity of microsomes in the absence of the soluble protein was 0.59 mmol ADP = $\mathrm{min^{-1}}$ $\mathrm{mg^{-1}}$. Results are means of three separate experiments. Inset shows the Hill plot ($\alpha_H = 0.97 \pm 0.11$).

For the present, we could not say that the results of our in vitro experiments actually represent the difference between shoots and roots in their sensitivities to auxin in terms of tissue growth. However, during auxin-stimulated shoot elongation an increase in H^+-ATPase of the plasma membrane and hyperpolarization of the plasma membrane have been reported to occur [20,21]. In this context, the in vitro effects of auxin at subcellular and molecular levels observed in the present work could be correlated with in vivo auxin effects in plants. If such is the case, it is tempting to speculate that alterations in distribution of SPF isoforms among different organs, tissues or regions of plant body under various physiological conditions may underlie the regulatory mechanisms for plant response to auxin.

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